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An Extended Fatty Liver Index to predict Nonalcoholic Fatty Liver Disease

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Abstract

Background: In clinical practice there is a strong interest in non-invasive markers of nonalcoholic fatty liver disease (NAFLD). We hypothesized that the fold-change of plasma triglycerides (TG) during a 2hr OGTT (fold-changeTG_{OGTT}), in concert with blood glucose and lipid parameters and the rs738409 C>G SNP in *PNPLA3* may improve the power of the widely used fatty liver index (FLI) to predict NAFLD.

Methods: In 330 individuals liver fat content was quantified by ^{1}H-magnetic resonance spectroscopy. Blood parameters were measured during fasting and after a 2hr OGTT. A subgroup of 213 individuals underwent these measurements before and after 9 months of a lifestyle intervention.

Results: The fold-changeTG_{OGTT} closely associated with liver fat content ($r=0.51$, $p<0.0001$), but predicted NAFLD less strong (ROC-AUC=0.75) than the FLI (ROC-AUC=0.79). Not only the fold-changeTG_{OGTT}, but also the 2hr blood glucose level and the rs738409 C>G SNP in *PNPLA3* independently associated with liver fat content and NAFLD. A novel index (extended FLI), generated from these parameters and the parameters of the FLI, considerably increased the power of the FLI to predict NAFLD (ROC-AUC from 0.79 to 0.86). The extended FLI also increased the predictive power of the FLI to predict the change of the liver fat content during a lifestyle intervention (N=213; from std. beta 0.23 to 0.29).

Conclusion: We provide novel data that the OGTT-derived fold-changeTG_{OGTT} and 2hr glucose level, together with the rs738409 C>G SNP in *PNPLA3,* allow the calculation of an extended FLI that considerably improves the power of the FLI to predict NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) has gained much attention in the recent years because of its high prevalence, amounting to more than 30% in the general population and to more than 70% in certain high risk groups, such as morbid obese individuals and patients with type 2 diabetes (1). NAFLD strongly associates not only with progressive hepatic, but also with cardiometabolic diseases and NAFLD is thought to be involved in the pathogenesis of cardiometabolic diseases, although the causative relationships have not been fully understood (2-12).

Diagnosis of NAFLD by the gold standard method, liver biopsy, is invasive and, therefore, not feasible in routine practice (13,14). Proton magnetic resonance spectroscopy (^{1}H-MRS) is considered the most accurate non-invasive method for measuring liver fat content (15,16). However, in addition to the high costs that limit its use, the respective infrastructure and knowledge that is needed to implement this technique are only available in a limited number of institutions. Therefore, ^{1}H-MRS is presently being applied mainly for research purposes. Routinely ultrasound is being used to diagnose NAFLD, but this technique has fair sensitivity only when liver fat content exceeds 20-30% (17). Consequently, there has been intense interest in blood markers that, alone or in combination with clinical parameters, would be able to identify patients with NAFLD. Accordingly, NAFLD or liver fat indexes were developed. However, some of them have a moderate predictive power and/or cannot be easily and widely used in the routine clinical practice, because they either involve several parameters that may not readily be measurable, or display great variability in their measurement, such as insulin, depending on the method that is being used (18-21). Furthermore, because there is a large variability in the decrease of liver fat content during a lifestyle intervention (22,23), it is important to investigate whether such indexes can predict the decrease of liver fat content during a lifestyle intervention.

It is, therefore, of great interest to identify readily measurable blood parameter that can either autonomously predict NAFLD, with relatively high sensitivity and specificity, or improve the predictive power of established indexes. For this purpose we intentionally tested only blood parameters that are commonly being measured, such as serum liver enzymes, lipids and lipoproteins, and that show no, or only little, variability between different labs. Furthermore, we studied the predictive power of the rs738409 C>G single nucleotide polymorphism (SNP) in *PNPLA3*, the strongest genetic determinant of NAFLD (24). Because it was recently shown that plasma triglycerides (TG) measured during an oral glucose tolerance test (OGTT) are closely related to abdominal obesity and insulin resistance (25), which strongly correlate with the liver fat content, we tested the circulating TGs not only in the fasted state, but also after a standard 2hr 75gr OGTT.

Methods

Subjects

Data of 330 Caucasians, 130 men and 200 women, from the southern part of Germany were analyzed. These individuals participated in the Tübingen Lifestyle Intervention Program (TULIP) (23,26). Individuals were included in the study when they fulfilled at least one of the following criteria: a family history of type 2 diabetes, a BMI > 27 kg/m^2, a previous diagnosis of impaired glucose tolerance and/or of gestational diabetes. They were considered healthy according to a physical examination and routine laboratory tests. **If diabetes was newly diagnosed based on the data from the screening visit subjects were included into the study.** They had no history of liver disease and did not consume more than two alcoholic drinks per day. Serum aminotransferase levels were lower than 2 times the upper limit of normal. From the 330 subjects who met the aforementioned requirements, mostly due to technical reasons, a subgroup of 213 (127 women and 86 men) had a complete data set of body fat distribution and liver fat content measurements using magnetic resonance techniques both, at baseline, and at follow-up and were included in the longitudinal analyses. Informed written consent was obtained from all participants and the Ethics Committee of the University of Tübingen had approved the protocol. The methods were carried out in accordance with the approved guidelines.

Lifestyle intervention

During the intervention subjects underwent individual dietary counseling and had up to ten sessions with a dietician. The aim was to reduce body weight, intake of calories and particularly intake of calories from fat to <30% (from saturated fat to <10%) of energy consumed and to increase intake of fibers to at least 15 gr/1000 kcal. During each visit participants presented a 3-day food diary and discussed the results with the dieticians. Individuals were asked to perform at least 3 hours of moderate

sports per week. Aerobic endurance exercise (e.g. walking, swimming) with an only moderate increase of the heart rate was encouraged. Participants were seen by the staff on a regular basis to ensure that these recommendations were accomplished.

Total body fat mass and body fat distribution

Body mass index was calculated as weight divided by the square of height (kg/m^2). Waist circumference was measured at the midpoint between the lateral iliac crest and lowest rib. Total body- and visceral fat mass were measured by MR tomography, with an axial T1-weighed fast spin echo technique with a 1.5 T whole-body imager (Magnetom Sonata, Siemens Medical Solutions) (16,27).

Liver fat content

Liver fat content was measured by localized ^{1}H-MR spectroscopy (15,26). NAFLD was defined as liver fat content >5.56% (15). Liver fat content measured by this method correlates well with histomorphometric findings (28,29).

Oral glucose tolerance test

All individuals underwent a 2hr 75gr OGTT. We obtained venous plasma samples at 0, 30, 60, 90 and 120 minutes for determination of plasma glucose and insulin. Blood glucose was determined using a bedside glucose analyzer (glucose-oxidase method; YSI, Yellow Springs Instruments, Yellow Springs, CO). Plasma insulin was determined using the ADVIA Centaur XP immunoassay system (Siemens Healthcare Diagnostics, Eschborn, Germany). Insulin sensitivity from the OGTT was calculated as proposed by Matsuda and DeFronzo (30). Furthermore, the homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated (31).

Analytical procedures

Plasma free fatty acids (FFAs), triglycerides (TGs), and total-, HDL- and low-density lipoprotein (LDL)-cholesterol were measured at 0 min, and FFA levels and TGs also at 2hr of the OGTT. Total cholesterol, HDL- and LDL-cholesterol, TGs, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) levels were measured using the ADVIA 1800 clinical chemical analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany). Plasma concentrations of total FFA were measured with an enzymatic method (WAKO Chemicals, Neuss, Germany) on the latter instrument. The SNP rs738409 C>G in *PNPLA3* was genotyped as previously described (32).

Calculations of liver fat indexes

For comparison purposes, among available liver fat indices, we calculated the Fatty Liver Index (FLI) as

$$[[e^{0.953 * \log_e(TG) + 0.139 * BMI + 0.718 * \log_e(GGT) + 0.053 * (\text{waist circumference}) - 15.745}] /$$

$$[1 + e^{0.953 * \log_e(TG) + 0.139 * BMI + 0.718 * \log_e(GGT) + 0.053 * (\text{waist circumference}) - 15.745}]] * 100 \quad (19)$$

and the Hepatic Steatosis Index (HSI) as

$$8 * ALT/AST + BMI(+2, \text{if type 2 diabetes; } +2, \text{if female}) \quad (21)$$

The NAFLD-Liver Fat Score (20) was not calculated, because it includes serum insulin, which, as mentioned above, displays great variability in its measurement depending on the method that is being used.

Statistical analyses

Data that were not normally distributed (Shapiro-Wilk W test) were logarithmically transformed to approximate a normal distribution. Differences in subjects' characteristics between the group with and without NAFLD were tested by student's t test for continuous and chi-square test for nominal parameters. The relationships of liver fat content with candidate blood markers in cross-sectional analysis were tested in univariate (Pearson correlation analyses) and multivariate linear regression models, with liver fat content set as the dependent variable and blood markers as independent variables, adjusted for gender, age, total- and visceral fat mass. The value of the candidate markers for diagnosing NAFLD was determined by calculating the area under the ROC-curve in nominal logistic regression analyses using univariate and multivariate models. In addition, the odds ratios (OR) for 1 SD increase of the candidate blood markers for having NAFLD were calculated using the same univariate and multivariate models. Differences between baseline and follow-up parameters were tested using the matched pairs t test. To determine the predictive effect of the candidate blood markers on the change of liver fat content during the intervention, multivariate regression analyses were performed. Logistic regression using the same independent covariates was applied to determine the OR for 1 SD increase of the candidate blood markers at baseline for responding to the intervention with a reduction of liver fat content. The statistical software package JMP 11.0 (SAS Institute Inc, Cary, NC, USA) was used. A p-value ≤ 0.05 was considered statistically significant.

Results

Cross-sectional analyses

Demographics, anthropometrics and metabolic characteristics of the subjects

The characteristics of the 330 subjects (130 men and 200 women) who had data at baseline are shown in table 1. **A total of 17 subjects were found to have newly diagnosed diabetes based on elevated fasting and/or 2 hr glucose values or elevated HbA1c values and 71 subjects had impaired glucose tolerance (IGT).** Subjects with NAFLD had more visceral fat mass and higher concentrations of serum liver enzymes. They also had higher glucose levels, higher 2hr FFAs, lower HDL- and higher LDL-cholesterol levels and higher fasting and 2hr TGs. Mean TG levels decreased during the OGTT, but significantly less strongly in subjects with NAFLD (table 1).

Associations of liver fat content with selected parameters

Besides the well established relationships of liver fat content with anthropometrics, glycemia and lipidemia, liver fat content was found to strongly correlate with liver enzymes, particularly GGT, fasting and 2hr TGs, 2hr glucose levels, the rs738409 C>G SNP in *PNPLA3*, the FLI, the HSI and with the fold-change of plasma triglycerides during the OGTT (2 hr/fasting; fold-changeTG_{OGTT}, $p<0.0001$) (supplemental table 1). In univariate and multivariate relationships the FLI and the fold-changeTG_{OGTT} emerged as the strongest determinants of liver fat content.

Determinants of NAFLD

We then explored the associations of the blood markers with NAFLD by calculating the OR of having NAFLD for 1 SD change of each of the parameters. In univariate analysis 1 SD increase (decrease for HDL-cholesterol) of all markers was significantly associated with the risk of having NAFLD (figure

1A). However, the 1 SD increase of fold-changeTG_{OGTT} (about 15% decreases of triglycerides during the OGTT) had an almost two-fold larger effect compared to most other markers (figure 1A). In a fully adjusted model, the relationships of all markers were attenuated, with the effect of HDL-cholesterol and fasting glucose and TGs being rendered non-significant (figure 1B). Nevertheless, the effect of fold-changeTG_{OGTT} remained greater than the effect of other markers (figure 1B). In particular, the OR for subjects having an increase of TGs during the OGTT (n=98, 29.7%) compared to those having a decrease of TGs for having NAFLD was 3.50 (95% CI, 2.12-5.78).

Next the accuracy of blood markers, the rs738409 C>G SNP in *PNPLA3* and the FLI and HSI for diagnosing NAFLD was determined by calculating the areas under the ROC curves. Among the blood markers the area under the ROC curve of fold-changeTG_{OGTT} was the largest, followed by the area under the ROC curve of GGT (table 2, univariate model). With gradual addition of gender, age, total fat mass and/or visceral fat mass in the model, the diagnostic accuracy increased; however, the area under the ROC curve of fold-changeTG_{OGTT} remained greater than that of any other single marker (table 2, models 1-4).With regard to the liver fat indexes, FLI showed a marked higher diagnostic accuracy (area under the ROC curve 0.79) compared to HSI (area under the ROC curve 0.70).

Changes of anthropometric and metabolic characteristics during the intervention

The duration of follow-up was 8.7 ± 1.8 (mean ± SD) months. The changes of the anthropometric and metabolic parameters during the intervention are shown in the table 3. The largest change was observed for liver fat content (-30.3%), followed by visceral fat mass (-14.4%) and total fat mass (-9.1%). A resolution of NAFLD was observed in 26 from 61 (42.6%) subjects. In contrast, 9 out of 152 (5.9%) of the subjects without NAFLD at baseline developed NAFLD at follow-up. Because in the latter group

body weight and fat compartments did not decrease significantly, we assume that they were not compliant with the recommendations of the study.

Predictors of the change of liver fat during the intervention

Logistic regression analysis was applied to calculate the OR for 1 standard deviation (SD) increase of the candidate blood markers at baseline for responding to the intervention with a decrease of liver fat content vs. non-responders. Again, fold-changeTG_{OGTT} at baseline displayed the highest OR (figure 2). A decrease of 15% (1 SD) of triglycerides during the baseline OGTT was associated with a 60% larger chance of improvement of liver fat content during the intervention.

When comparing the blood markers, the rs738409 C>G SNP in *PNPLA3*, the FLI and the HSI, the strongest predictive effect on the change of liver fat content was seen for fold-changeTG_{OGTT} at baseline (supplemental table 2). When adjusted for other possible confounders, HDL-cholesterol- and fasting triglyceride were not predictors of the change of liver fat content anymore. 2hr triglycerides still predicted the change of liver fat content, but the predictive effect of fold-changeTG_{OGTT} at baseline was stronger. Among the liver fat indexes, only the FLI at baseline predicted the change of liver fat content, but weaker than the fold-changeTG_{OGTT} (supplemental table 2).

Development of an Extended Fatty Liver Index to predict NAFLD

Based on the fact that in multivariate models the FLI, the fold-changeTG_{OGTT}, the 2hr glucose levels during the OGTT and the rs738409 C>G SNP in *PNPLA3* independently and strongly associated with liver fat content (all $p<0.0001$) and NAFLD [all $p<0.0001$, except for the rs738409 C>G SNP in *PNPLA3* ($p=0.002$) and the 2hr glucose levels ($p=0.0001$)] we generated an extended FLI by using these parameters. For this we first ran a multivariate model that included the parameters of the FLI, the

fold-changeTG_{OGTT}, the 2hr glucose levels during the OGTT and the rs738409 C>G SNP in *PNPLA3* (Table 4). In analogy to the FLI we then generated a formula for the extended FLI:

Extended FLI = (x / 1 + x) * 100

$x = e^{0.4508 * \log e (TG) + 0.0621 * BMI + 0.4022 * \log e (GGT) + 0.0454 * (\text{waist circumference}) + 4.8874 * (\text{fold-change}TG_{OGTT}) + 2.4134 * \log e (\text{2hr glucose}) - 1.1143 * (\text{rs738409 C>G SNP in } \textit{PNLA3; C=1 and XG=0}) - 19.1367}$

When compared to the FLI the extended FLI was found to have lower sensitivity, however, higher specificity, at the cut-off values 30 and 60. Furthermore, while at these cut-off values the negative predictive values were smaller for the extended FLI, the respective positive predictive values were larger (table 5). The power of the FLI to predict NAFLD (ROC-AUC 0.79) was increased to 0.86 when using the extended FLI (Figure 3). In this respect the inclusion of the parameters step-by-step revealed an improvement of the predictive power compared to the FLI (FLI+fold-changeTG_{OGTT} - ROC-AUC 0.82, p=0.062; FLI+fold-changeTG_{OGTT}+2hr glucose levels - ROC-AUC 0.84, p=0.0033) and compared to the FLI+fold-changeTG_{OGTT}+2hr glucose levels additional inclusion of the rs738409 C>G SNP in PNPLA3 further improved the predictive power (ROC-AUC 0.86, p=0.039). **After analyzing the predictive power of the extended FLI to predict NAFLD in groups of subjects with different stages of glycemia the predictive power was found to be higher in subjects with IGT (ROC-AUC 0.88) compared to subjects with NGT (ROC-AUC 0.83), while the predictive power was lowest in subjects with diabetes (ROC-AUC 0.78).** Finally, the extended FLI at baseline also increased the power of the FLI at baseline to predict the change of the liver fat content during a lifestyle intervention (N=213; from std. beta 0.23 to 0.29).

Discussion

Considering the hepatic and metabolic consequences of fat accumulation in the liver (2-12), there is a strong medical need for a simple, accurate and cost-effective biomarker of liver fat content. At least four such surrogate markers have been proposed, the SteatoTest (18), the FLI (19), the NAFLD-Liver Fat Score (NAFLD-LFS) (20) and the HSI (21). Among them, the FLI was shown to predict NAFLD in several populations (2,19,33-35), but mostly with ultrasound as the reference diagnostic tool. The same holds true, albeit in only a few populations, for the HSI. Recently, studies performed a head-to-head comparison and validation of these markers against the standard methods, liver biopsy (36) and ^{1}H-MRS (37). Both studies yielded qualitatively similar results. FLI, NAFLD-LFS and HSI displayed almost equal performance to identifying the presence of NAFLD, thus justifying their use as screening/surrogate markers. However, they did not show the same performance in quantifying liver fat content, and were not able to discriminate between mild, moderate and severe steatosis. Furthermore, it is unclear, whether these indexes are well suited for predicting the change of liver fat content in response to any pharmacological or non-pharmacological treatment of NAFLD.

In the present study we investigated whether single blood markers and the rs738409 C>G SNP in *PNPLA3* can improve the power of the established liver fat indexes to predict liver fat content, the presence of NAFLD, and the decrease of liver fat content during a lifestyle intervention. For this we first focused on single markers. We further restricted the number of candidate markers to only readily measured blood parameters, the methods of measurement of which are universally established and display the highest possible repeatability and lowest possible variability within the same or between several operators and labs.

After exploring the relationships of several routinely measured blood parameters with liver fat content, determined by the currently most accurate non-invasive method, ^{1}H-MRS (15,16), we found that liver

enzymes, particularly GGT, fasting- and 2hr glucose and TGs during an OGTT, as well as fold-changeTG_{OGTT}, strongly associated with liver fat content. We also found that among these parameters fold-changeTG_{OGTT} was the strongest determinant of liver fat content and the strongest predictor of NAFLD. Patients in whom TGs increased during the OGTT had a very high probability of having NAFLD, which was 3.5 times higher compared to patients in whom TGs decreased during the OGTT. Moreover, fold-changeTG_{OGTT} at baseline was able to predict the decrease of liver fat content during a lifestyle intervention, independently of the decrease of overall and visceral adiposity. This relationship was stronger than that of any other marker. A 15% decrease of TGs during an OGTT at baseline was associated with a 60% higher chance for the intervention to be successful, i.e. to result in a reduction of liver fat content.

In the cross-sectional analyses the FLI, but not the HSI, was stronger associated with liver fat content and was able to somewhat better predict NAFLD, compared to the fold-changeTG_{OGTT}. This was not unexpected, since fasting triglycerides are included in the formula of the FLI, and 3 additional determinants of liver fat content. Of note, when accounting for fold-changeTG_{OGTT}, BMI and waist circumference, the prediction of NAFLD increased to 0.80 and when also gender and age were taken into consideration, the area under the ROC curve further increased to 0.83. Moreover, the fold-changeTG_{OGTT} at baseline was a better predictor of the decrease of liver fat content during the lifestyle intervention, than the FLI at baseline. Thus, while the usefulness of the various liver fat indexes, particularly of the FLI, for diagnosing NAFLD, should not be questioned, fold-changeTG_{OGTT} could also be used as a screening parameter for identifying patients potentially having NAFLD, and may additionally represent a useful predictor of the change of liver fat content over time.

The precise mechanisms linking liver fat content with the change of triglycerides during the OGTT are unknown. In most cases, triglyceride levels decrease during an OGTT. This is thought to be a result of

the increase of insulin levels in response to the glucose load. High insulin levels suppress adipocyte lipolysis and FFA supply to the liver, ultimately leading to a decrease of VLDL production and triglyceride levels (38). Furthermore, insulin may suppress VLDL secretion independently of FFA influx through direct action on the liver. In addition, insulin directly enhances intravascular triglyceride hydrolysis by increasing the activity of lipoprotein lipase (LPL) and it stimulates hepatic low density lipoprotein-related protein-1 (LRP1), thereby increasing catabolism of triglyceride-rich VLDL and chylomicron particles (39,40). On the other hand, elevated glucose during the OGTT may counteract the effects of insulin by substrate competition with fatty acids, suppressing hepatic fatty acid oxidation and stimulating lipogenesis as well as VLDL production in the liver (41). It is plausible that the aforementioned effects of insulin would weaken with increasing insulin resistance, and eventually, the effects of hyperglycemia would prevail. This would lead to an increase of triglycerides levels, during the OGTT, preferentially in insulin resistant subjects with abdominal obesity, as observed in the present, and in other studies (25).

After having established that parameters of the FLI, as well as the fold-changeTG_{OGTT}, the 2hr glucose levels and the rs738409 C>G SNP in *PNPLA3* are strongly associated with NAFLD, we used these parameters to develop an extended FLI. This novel index was found to have a higher ROC-AUC for predicting NAFLD than the FLI. At the cut-off values 30 (lower values are thought to exclude NAFLD) and 60 (higher values are thought to prove NAFLD) the extended FLI was found to have lower sensitivity, however, higher specificity to predict NAFLD. Furthermore, while at these cut-off values the negative predictive values were smaller for the extended FLI compared to the FLI, the respective positive predictive values were larger. Thus, these data indicate that for a value $\geq$60 the extended FLI is better suited than the FLI to diagnose NAFLD, whereas, for a value <30 the FLI appears to be somewhat better suited to exclude NAFLD.

Our study has some limitations. We only studied Caucasians at risk for type 2 diabetes. Thus, there is an obvious need for validation of our findings in other ethnic groups and in patients with type 2 diabetes. Particularly in the latter group, other biomarkers, including FLI and HSI, showed a relatively low ability for predicting NAFLD (42). Furthermore, although there was great variability and range in nearly all anthropometric and metabolic parameters across the participants of the present study, our population included no subjects with extremely high liver fat content. Since the accuracy of other biomarkers for diagnosing such high levels of liver fat content is low (36), the findings of the present study may not fully apply to subjects with this phenotype. Nevertheless, the fact that in an Italian population with biopsy-proven NAFLD (personal communication with A. Gastaldelli and E. Bugianesi; N=23, mean age=42 years) the fold-changeTG_{OGTT} measured during a 2hr 75g OGTT was found to be 1.01, which is very similar to the ratio in the German population with NAFLD, supports that the fold-changeTG_{OGTT} could be used to estimate the risk of NAFLD. Finally, the calculation of our proposed extended FLI requires the performance of a 2hr OGTT and the determination of the rs738409 C>G SNP in *PNPLA3*. However, as diagnosis of prediabetes and diabetes becomes more important today, particularly in subjects with suspected NAFLD, and OGTT-derived parameters were found to predict advanced liver damage (43), OGTTs will be more often incorporated in the clinical setting. **Because the predictive power of the extended FLI was highest in subjects with IGT there is support for calculating this index particularly in this high risk population, in whom an OGTT has been performed.** Furthermore, genotyping of the most important genetic determinant of NAFLD, NASH and possibly fibrosis, may also become a routine diagnostic approach in clinical practice. **Nevertheless, to date it may be difficult to use the extended FLI in clinical practice only for the prediction of liver fat content. However, if in future studies the extended FLI may also turn out to be a good**

predictor of fibrosis, it may become a more widely used non-invasive estimate of advanced stages of NAFLD.

In conclusion, we provide novel data that the OGTT-derived fold-changeTG_{OGTT} and 2hr glucose levels, together with the rs738409 C>G SNP in *PNPLA3*, allow the calculation of an extended FLI that considerably improves the power of the FLI to predict NAFLD.

Contribution statement

Study concept and design: NS, LS and HUH. Acquisition of data: KK, IR, JM, FS, AF, NS AG, EB. Analysis and interpretation of data: KK, NS, AG, EB, MBS and HUH. Drafting of the manuscript: KK and NS. Critical revision of the manuscript for important intellectual content: KK, IR, HS, AF, LS, AP, AG, EB, MBS, HUH, NS. Statistical analysis: KK, AF, NS. Administrative, technical, and material support: KK, IR, HS, JM, FS, AF, AP, HUH, NS. Study supervision: NS, HUH.

Additional Information

Competing financial interests

The authors have no financial interests with this work.

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Table 1 Subjects' demographic and metabolic characteristics.

	All	Subjects without NAFLD	Subjects with NAFLD	p
Demographics				
Gender (females / males)	200 / 130	150 / 75	50 / 55	0.001*
Age (years)	45.5 ± 0.7	44.2 ± 0.8	48.2 ± 1.1	0.002
Body composition				
Body weight (kg)	86.39 ± 0.93	82.92 ± 1.06	93.82 ± 1.63	<0.0001
Body mass index ($kg \cdot m^{-2}$)	29.50 ± 0.27	28.34 ± 0.31	31.99 ± 0.45	<0.0001
Waist circumference (cm)	96.5 ± 0.7	92.7 ± 0.8	104.6 ± 1.1	<0.0001
Total body fat (kg)	26.16 ± 0.58	24.57 ± 0.69	29.59 ± 1.01	<0.0001
Subcutaneous abdominal fat (kg)	11.35 ± 0.27	10.57 ± 0.32	13.04 ± 0.49	<0.0001
Visceral fat (kg)	2.95 ± 0.10	2.37 ± 0.10	4.20 ± 0.18	<0.0001
Liver fat (%)	5.80 ± 0.35	2.38 ± 0.11	13.14 ± 0.64	<0.0001
Fatty liver (n)	105 (31.82%)		-----	-----
Metabolic characteristics				
ALT (U/L)	28.77 ± 1.07	25.95 ± 0.99	34.93 ± 2.51	<0.0001
AST (U/L)	24.86 ± 0.61	23.00 ± 0.50	28.75 ± 1.49	<0.0001
GGT (U/L)	27.34 ± 1.53	23.43 ± 1.65	35.79 ± 3.14	<0.0001
Fasting glucose (mM)	5.26 ± 0.03	5.18 ± 0.03	5.44 ± 0.06	<0.0001
2 h glucose (mM)	7.07 ± 0.10	6.75 ± 0.11	7.75 ± 0.21	<0.0001
Fasting insulin (pM)	62.1 ± 2.3	52.5 ± 2.1	82.4 ± 4.9	<0.0001
2 h insulin (pM)	498.8 ± 21.3	395.9 ± 20.4	717.3 ± 43.3	<0.0001
NGT/IGT/Dia	**242/71/17**	**184/36/5**	**58/35/12**	**<0.0001***
Fasting free fatty acids (µM)	660 ± 15	658 ± 18	665 ± 24	0.66
2 h free fatty acids (µM)	77 ± 4	66 ± 4	95 ± 6	<0.0001
Fasting TG (mg/dl)	112.71 ± 4.45	101.81 ± 4.66	135.56 ± 9.36	<0.0001
2hr TG (mg/dl)	104.89 ± 4.26	90.75 ± 4.33	134.67 ± 8.96	<0.0001
Fold-changeTG_{OGTT} (2hr TG/fasting TG)	0.912 ± 0.008	0.872 ± 0.010	0.997 ± 0.011	<0.0001
$AUC_{0\text{-}2hr}$ TG (mg/dl)	220.25 ± 8.72	196.26 ± 9.20	270.32 ± 17.95	<0.0001
Total cholesterol (mg/dl)	194.7 ± 2.0	193.3 ± 2.5	197.8 ± 3.1	0.15
HDL-cholesterol (mg/dl)	52.3 ± 0.7	54.2 ± 0.9	48.2 ± 1.1	<0.0001

LDL-cholesterol (mg/dl)	123.2 ± 1.7	120.3 ± 2.0	129.3 ± 2.9	0.008
HOMA-IR index	1.99 ± 0.08	1.65 ± 0.07	2.72 ± 0.17	<0.0001
Insulin sensitivity$_{OGTT}$ (AU)	12.73 ± 0.40	14.62 ± 0.46	8.72 ± 0.46	<0.0001
rs738409 C>G SNP in *PNPLA3*				
CC	188	137	51	
XG	142	88	54	0.03*
Fatty Liver Index	50.68 ± 1.60	41.40 ± 1.82	70.75 ± 2.12	<0.0001
Hepatic Steatosis Index	39.91 ± 0.36	38.55 ± 0.42	42.76 ± 0.58	<0.0001

p for the difference between NAFLD and non-NAFLD patients (unadjusted, t-test) * chi-square
Values represent unadjusted means ± SE (standard error). For statistical analyses, non-normally distributed parameters were log transformed. **NGT: normal glucose tolerance; IGT: impaired glucose tolerance; Dia: diabetes**; AUC: area under the curve; AU: arbitrary units

Table 2 Accuracy of selected blood markers for diagnosing nonalcoholic fatty liver disease (area under the Receiver Operating Characteristic curve)

Predictor	Univariate	Model 1[1]	Model 2[2]	Model 3[3]	Model 4[4]
ALT	0.6686	0.6988	0.7572	0.8131	0.8139
AST	0.6511	0.6883	0.7605	0.8119	0.8124
GGT	0.7102	0.7161	0.7712	0.8140	0.8143
HDL-cholesterol	0.6531	0.6956	0.7562	0.8080	0.8091
Fasting glucose	0.6243	0.6701	0.7482	0.8086	0.8089
2hr glucose	0.6534	0.7042	0.7787	0.8219	0.8215
rs738409 C>G SNP in *PNPLA3*[#]	0.5616	0.6590	0.7567	0.8190	0.8185
Fasting TG	0.6582	0.6966	0.7688	0.8128	0.8118
2hr TG	0.7147	0.7328	0.7879	0.8191	0.8191
Fold-changeTG_{OGTT} (2hr/fasting)	0.7508	0.7665	0.8073	0.8329	0.8330
Fatty Liver Index	0.7912	0.7958	0.7944	0.8214	0.8258
Hepatic Steatosis Index	0.7017	0.7473	0.7511	0.8037	0.8080

[1]**Model 1:** Adjusted for gender and age

[2]**Model 2:** Adjusted for gender, age and total fat mass

[3]**Model 3**: Adjusted for gender, age and visceral fat mass

[4]**Model 4**: Adjusted for gender, age, total adipose fat mass and visceral fat mass

[#]dominant model (CC vs XG)

Table 3 Demographic and metabolic characteristics of subjects who participated in the lifestyle intervention.

Parameter	All subjects			Subjects without NAFLD at baseline			Subjects with NAFLD at baseline		
	Baseline	Follow-up		Baseline	Follow-up	p	Baseline	Follow-up	p
Gender (females / males)	127 / 86			103 / 49			24 / 37		
Age (years)	45.7 ± 0.8	46.4 ± 0.8	<0.0001	45.1 ± 0.9	45.8 ± 0.9	<0.0001	47.3 ± 1.4	48.1 ± 1.4	0.0015
Body weight (kg)	85.6 ± 1.1	83.1 ± 1.1	<0.0001	82.2 ± 1.2	80.1 ± 1.2	0.032	94.1 ± 2.0	90.7 ± 2.1	0.024
Body mass index ($kg \cdot m^{-2}$)	29.04 ± 0.32	28.19 ± 0.31	<0.0001	27.99 ± 0.36	27.27 ± 0.35	0.031	31.64 ± 0.54	30.47 ± 0.54	0.024
Waist circumference (cm)	95.9 ± 0.9	92.6 ± 0.8	<0.0001	92.2 ± 1.0	89.2 ± 0.9	0.008	105.2 ± 1.5	100.9 ± 1.5	0.076
Total body fat (kg)	25.40 ± 0.68	23.08 ± 0.67	0.013	24.18 ± 0.80	22.13 ± 0.78	0.10	28.43 ± 1.21	25.45 ± 1.27	0.024
Subcutaneous abdominal fat (kg)	10.86 ± 0.31	10.16 ± 0.31	0.0015	10.24 ± 0.36	9.71 ± 0.35	0.036	12.40 ± 0.60	11.27 ± 0.60	0.008
Visceral fat (kg)	2.92 ± 0.13	2.52 ± 0.12	<0.0001	2.36 ± 0.12	2.00 ± 0.11	<0.0001	4.33 ± 0.24	3.82 ± 0.27	0.0002
Liver fat $_{MRS}$ (%)	5.34 ± 0.42	3.72 ± 0.28	0.0005	2.20 ± 0.11	1.98 ± 0.14	0.012	13.18 ± 0.83	8.05 ± 0.64	0.033
NAFLD	61 (28.6%)	44 (20.7%)	<0.0001	0 (0%)	9 (5.9%)		61 (100%)	35 (57.4%)	
ALT (U/L)	27.65 ± 1.06	24.08 ± 1.03	<0.0001	25.76 ± 1.25	21.80 ± 1.13	<0.0001	32.33 ± 1.86	29.85 ± 2.07	0.024
AST (U/L)	25.14 ± 0.80	23.27 ± 0.53	0.047	23.09 ± 0.61	22.42 ± 0.51	0.32	30.11 ± 2.20	25.70 ± 1.37	0.12
GGT (U/L)	26.45 ± 1.91	25.47 ± 2.05	0.11	21.59 ± 1.79	22.32 ± 2.39	0.54	38.56 ± 4.64	33.40 ± 3.83	0.16
Fasting glucose (mM)	5.26 ± 0.04	5.18 ± 0.04	0.022	5.18 ± 0.04	5.14 ± 0.04	0.48	5.45 ± 0.08	5.29 ± 0.08	0.16
2hr glucose (mM)	6.93 ± 0.11	6.71 ± 0.11	0.013	6.64 ± 0.11	6.59 ± 0.13	0.041	7.67 ± 0.22	7.02 ± 0.23	0.56
Fasting insulin (pM)	59.1 ± 2.4	52.3 ± 2.2	0.0002	51.0 ± 2.2	45.6 ± 2.0	0.11	79.1 ± 5.2	69.0 ± 5.2	0.23
2hr insulin (pM)	491.1 ± 26.2	429.2 ± 27.4	0.001	399.5 ± 25.0	372.7 ± 24.9	0.08	717.9 ± 57.5	570.1 ± 70.3	0.0496
Fasting free fatty acids (μM)	667 ± 18	600 ± 14	0.003	674 ± 23	612 ± 16	0.99	648 ± 25	570 ± 30	0.07
2hr free fatty acids (μM)	78 ± 5	77 ± 12	0.0004	68 ± 6	80 ± 17	0.23	130 ± 9	70 ± 6	0.64

Fasting TG (mg/dl)	112.8 ± 5.9	107.0 ± 5.9	0.006	104.8 ± 6.3	96.3 ± 6.0	0.018	132.9 ± 13.3	134.2 ± 13.7	0.029
2rh TG (mg/dl)	103.7 ± 5.6	94.5 ± 5.4	<0.0001	92.1 ± 5.9	81.6 ± 5.6	0.004	132.4 ± 12.5	127.1 ± 11.9	0.014
Fold-changeTG_{OGTT} (2hr/fasting)	0.90 ± 0.01	0.87 ± 0.01	0.0007	0.86 ± 0.01	0.83 ± 0.01	0.25	1.00 ± 0.02	0.95 ± 0.02	0.17
$AUC_{0\text{-}120}$ TG (mg/dl)	219.0 ± 11.6	202.2 ± 11.1	0.001	200.3 ± 12.5	179.2 ± 11.5	0.008	265.4 ± 25.2	260.4 ± 24.8	0.023
Total cholesterol (mg/dl)	193.5 ± 2.6	191.0 ± 2.7	0.16	193.1 ± 3.2	189.3 ± 3.1	0.07	194.4 ± 4.0	195.5 ± 5.1	0.09
HDL-cholesterol (mg/dl)	52.7 ± 0.9	52.7 ± 0.9	0.38	54.6 ± 1.1	54.5 ± 1.2	0.45	48.07 ± 1.5	48.07 ± 1.4	0.73
LDL-cholesterol (mg/dl)	121.5 ± 2.1	117.6 ± 2.3	0.013	119.2 ± 2.5	115.1 ± 2.7	0.16	127.2 ± 3.7	123.8 ± 4.4	0.57
HOMA-IR index	1.89 ± 0.08	1.64 ± 0.07	<0.0001	1.61 ± 0.08	1.43 ± 0.07	0.11	2.60 ± 0.18	2.19 ± 0.17	0.18
Insulin $sensitivity_{OGTT}$ (AU)	12.96 ± 0.47	14.80 ± 0.54	<0.0001	14.51 ± 0.54	16.25 ± 0.64	0.19	9.11 ± 0.69	11.18 ± 0.83	0.013
Fatty Liver Index	48.18 ± 2.05	41.98 ± 2.04	<0.0001	39.26 ± 2.23	33.49 ± 2.15	<0.0001	70.39 ± 3.01	63.35 ± 3.44	0.002
Hepatic Steatosis Index	39.10 ± 0.40	38.31 ± 0.56	0.001	38.05 ± 0.47	37.21 ± 0.68	0.004	41.62 ± 0.65	41.45 ± 0.83	0.059

AUC: area under the curve; AU: arbitrary units

Table 4 Multivariate linear regression model for the prediction of NAFLD in cross-sectional data

Parameter	Estimate	SE	Wald Chi-Square	p
Intercept	-19.1367	2.4354	61.7439	<.0001
BMI	0.0621	0.0519	1.4324	0.2314
Waist circumference	0.0454	0.0217	4.3530	0.0369
Log fasting TG	0.4508	0.3331	1.8314	0.1760
Log GGT	0.4022	0.2425	2.7506	0.0972
Fold-changeTG_{OGTT} (2hr/fasting)	4.8874	1.2676	14.8668	0.0001
Log 2hr glucose	2.4134	0.6216	15.0750	0.0001
rs738409 C>G SNP in *PNPLA3*[#]	-1.1143	0.3104	12.8876	0.0003

[#]dominant model (CC vs XG)

Table 5 Prediction of NAFLD in cross-sectional data

	Sensitivity	False +	Specificity	False -	PPV	NPV
FLI>=10	99.07	87.00	13.00	0.93	35.33	96.67
FLI>=20	97.20	69.96	30.04	2.80	40.00	95.71
FLI>=30	94.39	56.95	43.05	5.61	44.30	94.12
FLI>=40	88.79	47.98	52.02	11.21	47.03	90.63
FLI>=50	84.11	38.57	61.43	15.89	51.14	88.96
FLI>=60	72.90	26.46	73.54	27.10	56.93	84.97
FLI>=70	53.27	19.73	80.27	46.73	56.44	78.17
FLI>=80	36.45	10.31	89.69	63.55	62.90	74.63
FLI>=90	26.17	3.59	96.41	73.83	77.78	73.13
Extended FLI >=10	96.26	56.95	43.05	3.74	44.78	96.00
Extended FLI >=20	92.52	34.53	65.47	7.48	56.25	94.81
Extended FLI >=30	77.57	24.66	75.34	22.43	60.14	87.50
Extended FLI >=40	69.16	18.39	81.61	30.84	64.35	84.65
Extended FLI >=50	60.75	13.00	87.00	39.25	69.15	82.20
Extended FLI >=60	48.60	8.07	91.93	51.40	74.29	78.85
Extended FLI >=70	30.84	3.14	96.86	69.16	82.50	74.48
Extended FLI >=80	19.63	2.24	97.76	80.37	80.77	71.71
Extended FLI >=90	8.41	1.35	98.65	91.59	75.00	69.18

Figure legends

Figure 1

Odds ratios (OR) for 1 standard deviation (SD) higher baseline alanine transferase (ALT), aspartate transferase (AST), gamma-glutamyltranspeptidase (GGT), HDL-cholesterol, fasting- and 2hr glucose triglycerides (TG) during a 2hr 75g oral glucose tolerance test (OGTT), and fold-change of TGs during the OGTT (2hr/fasting; fold-changeTG_{OGTT}) for having non-alcoholic fatty liver disease (NAFLD) in univariate analyses (panel A) and in multivariate analyses with additional adjustment for gender, age, total adipose tissue and visceral adipose tissue mass (panel B).

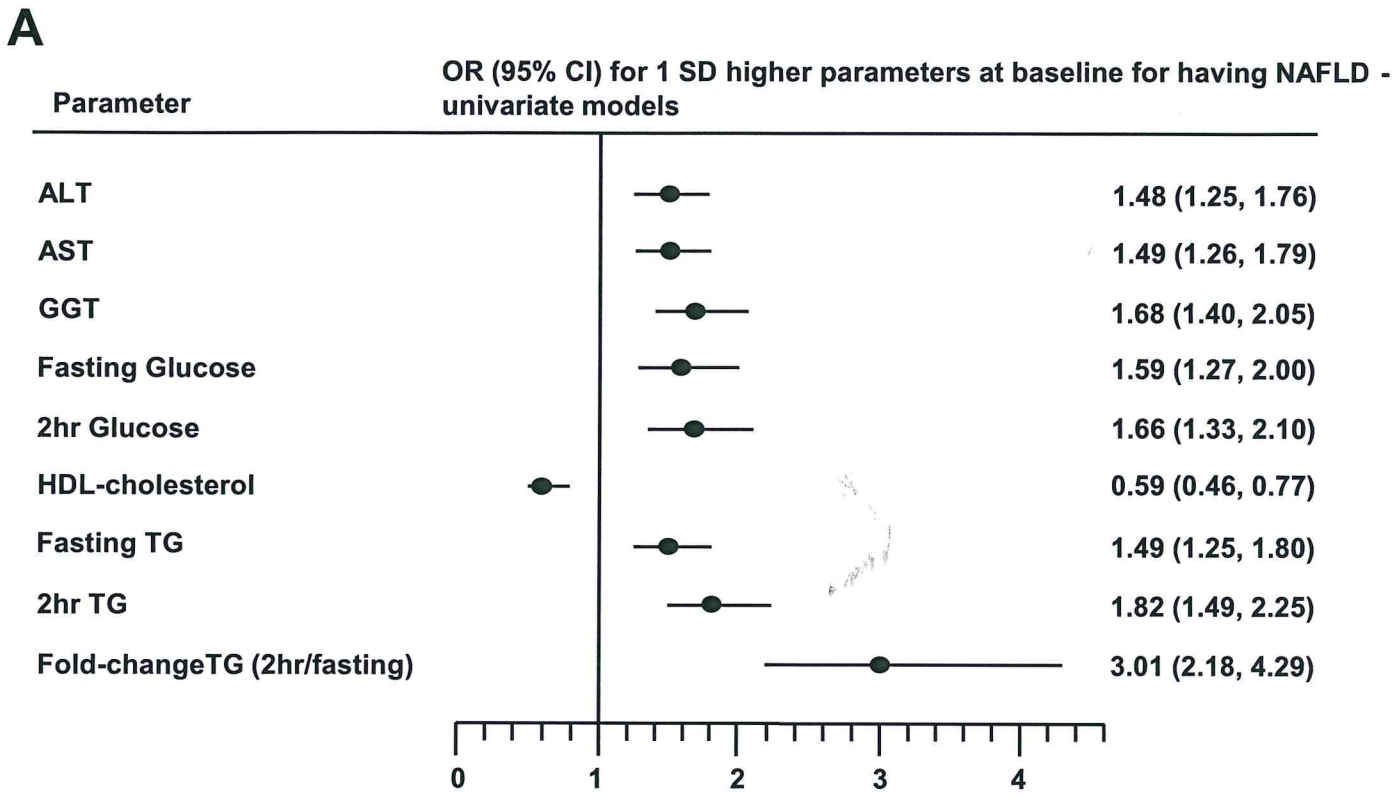
Figure 2

Odds ratios (OR) for 1 standard deviation (SD) higher baseline alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), HDL-cholesterol, fasting- and 2hr glucose and triglycerides (TG) during a 2hr 75g oral glucose tolerance test (OGTT), and fold-change of TGs during the OGTT (2hr/fasting; fold-changeTG_{OGTT}) for responding to the intervention in terms of liver fat content, i.e. for reducing vs. increasing or maintain the same liver fat content. Models were adjusted for liver fat content at baseline (panel A) or, additionally, for gender, age, total adipose tissue and visceral adipose tissue mass at baseline (panel B).

Figure 3

Prediction of NAFLD by calculating the fatty liver index (FLI) and the newly developed extended FLI in *cross-sectional* data.

Figure 1


A
Parameter
OR (95% CI) for 1 SD higher parameters at baseline for having NAFLD - univariate models
ALT
AST
GGT
Fasting Glucose
2hr Glucose
HDL-cholesterol
Fasting TG
2hr TG
Fold-changeTG (2hr/fasting)
1.48 (1.25, 1.76)
1.49 (1.26, 1.79)
1.68 (1.40, 2.05)
1.59 (1.27, 2.00)
1.66 (1.33, 2.10)
0.59 (0.46, 0.77)
1.49 (1.25, 1.80)
1.82 (1.49, 2.25)
3.01 (2.18, 4.29)
0
1
2
3
4

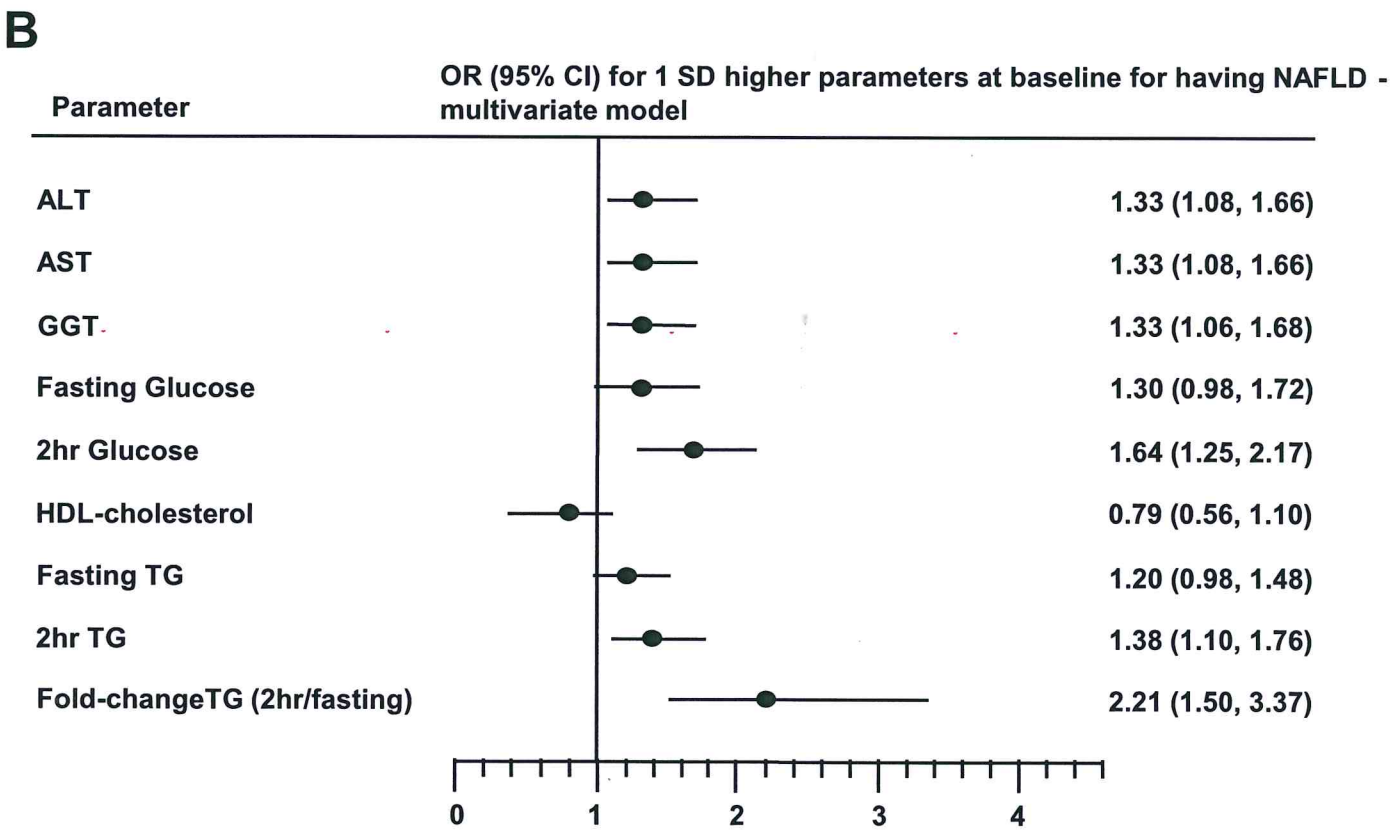
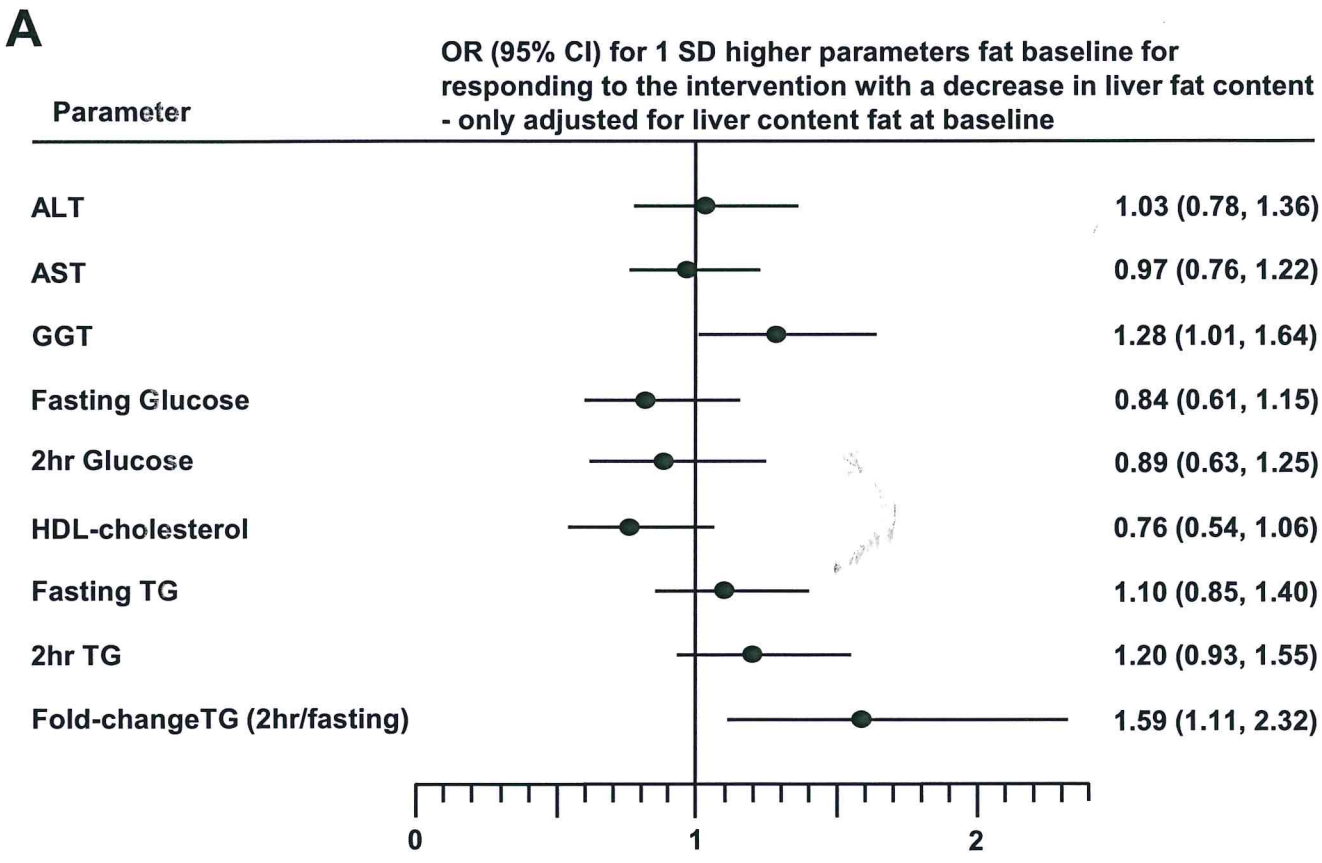

B
Parameter
OR (95% CI) for 1 SD higher parameters at baseline for having NAFLD - multivariate model
ALT
AST
GGT
Fasting Glucose
2hr Glucose
HDL-cholesterol
Fasting TG
2hr TG
Fold-changeTG (2hr/fasting)
1.33 (1.08, 1.66)
1.33 (1.08, 1.66)
1.33 (1.06, 1.68)
1.30 (0.98, 1.72)
1.64 (1.25, 2.17)
0.79 (0.56, 1.10)
1.20 (0.98, 1.48)
1.38 (1.10, 1.76)
2.21 (1.50, 3.37)
0
1
2
3
4

Figure 2


A
Parameter
OR (95% CI) for 1 SD higher parameters fat baseline for responding to the intervention with a decrease in liver fat content - only adjusted for liver content fat at baseline
ALT
AST
GGT
Fasting Glucose
2hr Glucose
HDL-cholesterol
Fasting TG
2hr TG
Fold-changeTG (2hr/fasting)
1.03 (0.78, 1.36)
0.97 (0.76, 1.22)
1.28 (1.01, 1.64)
0.84 (0.61, 1.15)
0.89 (0.63, 1.25)
0.76 (0.54, 1.06)
1.10 (0.85, 1.40)
1.20 (0.93, 1.55)
1.59 (1.11, 2.32)
0
1
2

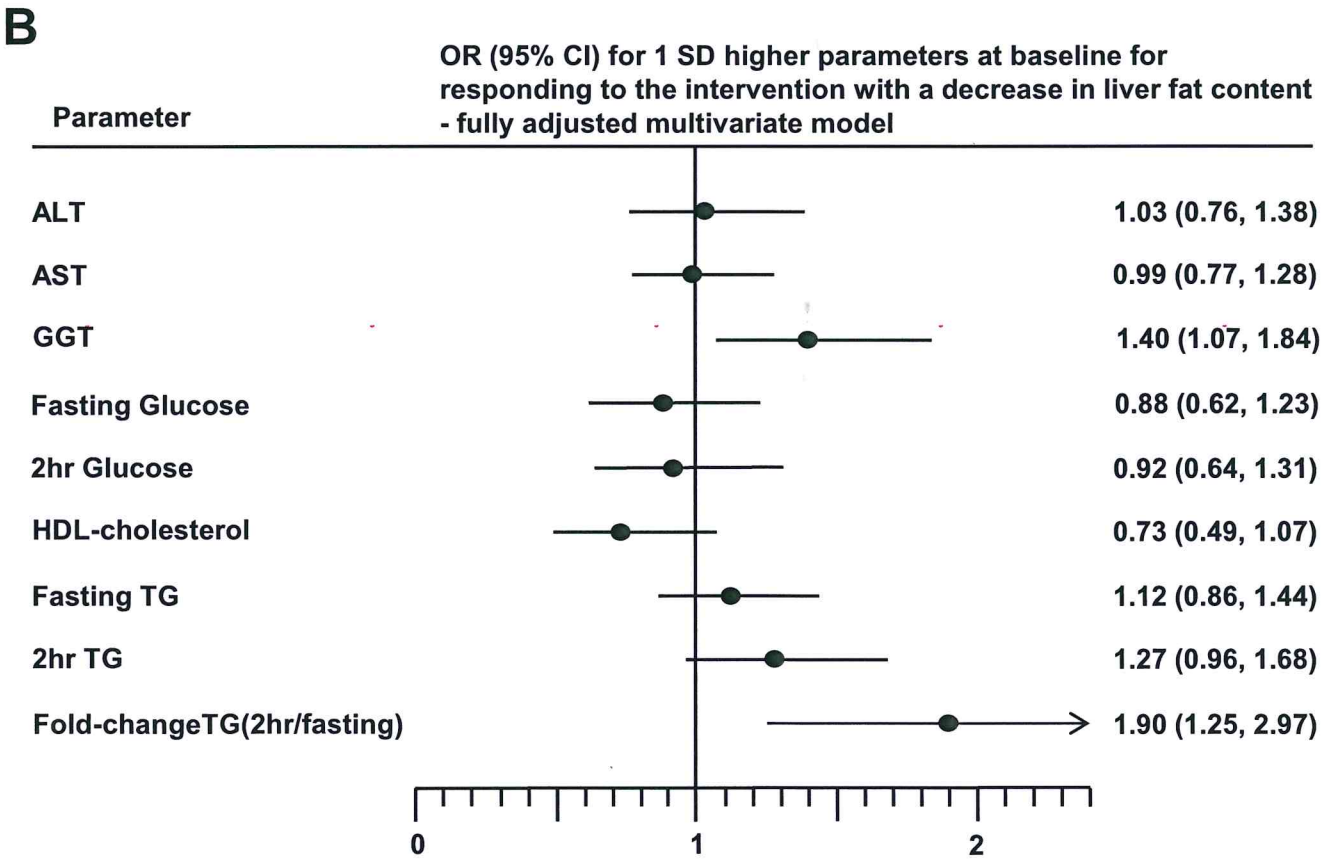
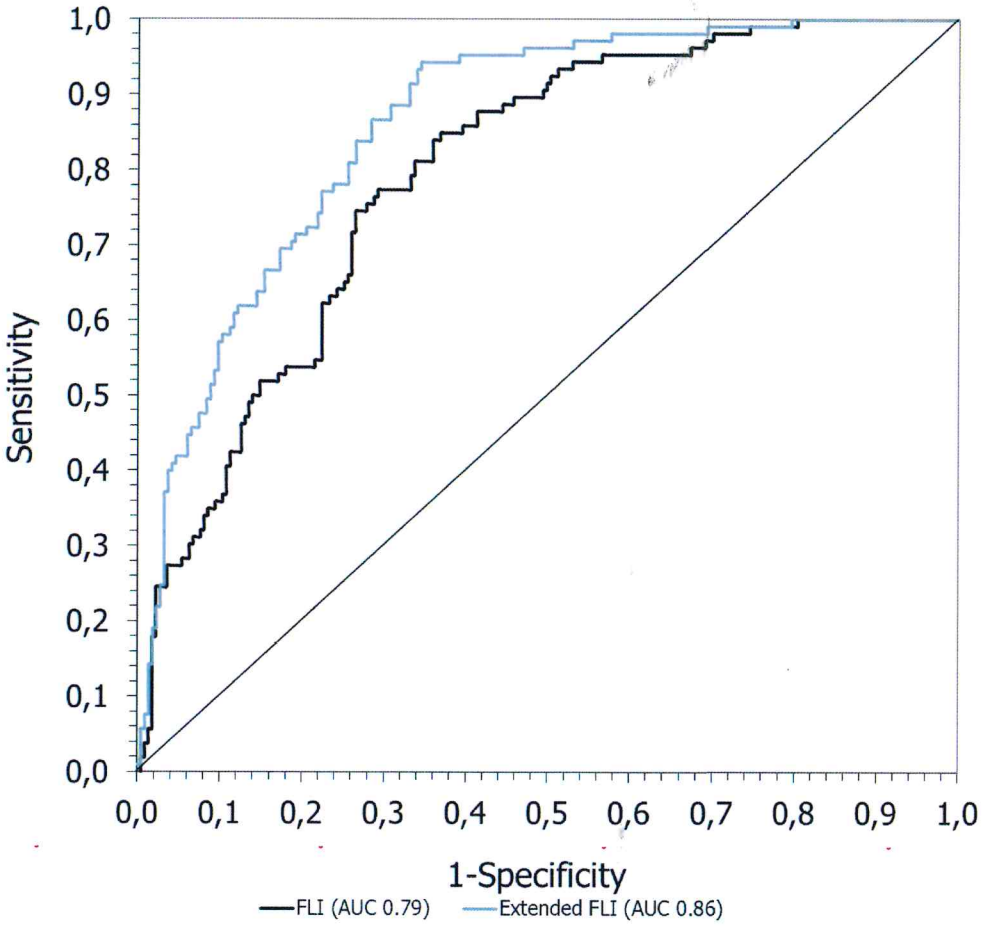

B
Parameter
OR (95% CI) for 1 SD higher parameters at baseline for responding to the intervention with a decrease in liver fat content - fully adjusted multivariate model
ALT
AST
GGT
Fasting Glucose
2hr Glucose
HDL-cholesterol
Fasting TG
2hr TG
Fold-changeTG(2hr/fasting)
1.03 (0.76, 1.38)
0.99 (0.77, 1.28)
1.40 (1.07, 1.84)
0.88 (0.62, 1.23)
0.92 (0.64, 1.31)
0.73 (0.49, 1.07)
1.12 (0.86, 1.44)
1.27 (0.96, 1.68)
1.90 (1.25, 2.97)
0
1
2

Figure 3


1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Sensitivity
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
1-Specificity
FLI (AUC 0.79)
Extended FLI (AUC 0.86)